United States Department of Agriculture
Agricultural Marketing Service, Science & Technology
Microbiological Data Program

SOP No.: MDP-MTH-01		Page 1 of 10
Title: *Escherichia coli* MPN Method		
Revision: 03	Replaces: 02	Effective: 03/01/04

1. Purpose & Principle:

To provide a standard procedure based on the Most Probable Number (MPN) for performing analysis of *Escherichia coli (E. coli)* using Lauryl Sulfate Tryptose Broth (LST), in the presence of 4-methylumbelliferyl β-D-glucuronide (MUG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Over 95% of the *E. coli* strains (including non-gas producing) produce β-D-glucuronidase (GUD), an enzyme that cleaves MUG to release 4-methylumbelliferone (MU). The presence of *E. coli* is identified by detecting MU which fluoresces blue on exposure to ultraviolet (UV) light at a long wavelength. The test also detects total coliforms based on the presence of β-galactosidase. This enzyme activity is detected by its ability to cleave X-Gal into an 5-bromo-4-chloro-indoxyl intermediate, which upon oxidation results in a water-insoluble blue compound.

2. Scope:

This standard operating procedure (SOP) shall be followed by all laboratories conducting microbiological studies for MDP, including support laboratories conducting non-routine activities that may impact the program.

3. Outline of Procedure:

4. References:

4.1 Robert Blodgett. 2001. Most Probable Number from Serial Dilutions. Appendix 2. FDA. BAM. http://www.cfsan.fda.gov/~ebam/bam-a2.html

4.2 USDA, FSIS, Microbiology Laboratory Guidebook. SOP No: MLG Appendix 2.02. Most Probable Number Tables. Revision: 02. Effective: 7/3/03 http://www.fsis.usda.gov/ophs/microlab/Appendix2.02.pdf

4.3 Peter Feng, Stephen D. Weagant, Michael A. Grant. 2002. Enumeration of *Escherichia coli* and the Coliform Bacteria. Chapter 4. FDA. BAM. http://www.cfsan.fda.gov/~ebam/bam-4.html

4.4 Peter Feng and Stephen D. Weagant. 2002. Diarrheagenic *Escherichia coli*. Chapter 4A. FDA. BAM. http://www.cfsan.fda.gov/~ebam/bam-4a.html

4.5 ColiComplete, BioControl Systems, Inc. Bothell, WA 98011 and AOAC Official method 992-30. http://www.biocontrolsystems.com/products/colicomplete.html

4.6 SOPs MDP-SHIP-0, MDP-SHIP-02, and MDP-MTH-04

5. Specific Procedures:

5.1 Equipment and Materials

5.1.1 Incubator, 35 ± 2°C

5.1.2 Petri dishes, sterile, 15 x 100 mm

5.1.3 Pipettes, sterile with cotton plug, graduated in 0.1 mL units. Total volume 1.0 mL and 10.0 mL or any fixed volume pipettor capable of delivering the appropriate volume. Pipette aids such as fillers, dispensers or pipettors are required for transferring viable cultures.

5.1.4 Test tubes: 20 x 150 mm or of sufficient capacity to allow space for adequate mixing

5.1.5 Inoculation loops (~3 mm), sterile wooden applicator sticks, and inoculating needle

5.1.6 Thermometers, one immersion type, or digital probe with 0.1° subdivisions and one thermometer with a range that includes 35°C. Both thermometers calibrated to a standard thermometer that is certified by a National Institute of Standards and Technology (NIST) thermometer

5.1.7 ColiComplete discs (BioControl Systems, Inc.)

5.1.8 VITEK (bioMerieux)

5.1.9 Cryovials, sterile

5.1.10 Sterile forceps or device to dispense discs

5.2 Media and Reagents

5.2.1 Levine's eosine-methylene blue (L-EMB) agar

5.2.2 Eluent: Buffered peptone water (BPW) + 0.1% Tween 80

5.2.3 Double strength (DS) and single strength (SS) LST in 10 mL volumes

5.2.4 Non-specific rich broth (lactose broth, brain heart infusion broth, Luria broth, etc.)

5.2.5 Glycerol (1,2,3-Propanetriol), ACS Grade, sterile

5.2.6 *E. coli,* Coli Genetic Stock Center (CGSC) strain # 7372, F^-, λ^-, *trpC*3117::Tn10kan, *rph-1*), positive control

5.2.7 *Enterobacter aerogenes (E. aerogenes)*, negative control

5.3 Control Methods

5.3.1 Procedure for preparing organisms for use as positive and negative controls

5.3.1.1 On the day prior to testing a group of produce samples, inoculate each of the control strains in a non-specific rich broth and incubate at 35±2°C overnight:

5.3.1.2 On the day of testing, inoculate 3 vials with small volumes of a non-specific rich broth with the organisms and incubate at 35±1°C to approximately 0.5 McFarland turbidity (faintly turbid visually). Use this suspension to set up the quality control tubes during testing.

5.3.2 Use the following controls in the setup:

5.3.2.1 Negative Media Controls: 10mL BPW + 0.1% Tween 80 in 10mL DS LST and 1mL BPW + 0.1% Tween 80 in 10mL SS LST

5.3.2.2 *E. coli* Positive Control: 10mL *E. coli* culture suspension in 10mL DS LST and 1mL *E. coli* culture suspension in 10mL SS LST

5.3.2.3 *E. aerogenes* Negative Control: 10mL *E. aerogenes* culture suspension in 10mL DS LST and 1mL *E. aerogenes* culture suspension in 10mL SS LST

5.3.2.4 Produce Culture Control: After saving a minimum of 15mL (in case re-testing of the sample is required) add 1 mL *E. coli* culture from 5.3.1.2 (above) and to a single produce sample

chosen at random after eluate is inoculated into test cultures. The positive control organism for MDP-MTH-04 should be added if the same positive produce sample is being used for that procedure. Gently mix produce by hand – do not use shaker; 10 mL of the produce control is inoculated in 10 mL DS LST and 1mL is inoculated in 10 mL SS LST.

5.3.2.5 Incubate, subculture, and test the control cultures along with the MDP produce test samples. Include controls with any confirmation steps performed on samples.

5.3.3 Compare the results obtained at each step of the cultural process with the expected results for each control. Investigate any results which fail to conform as expected and identify the source of unexpected results. If results of QC cultures fail to conform, refer to MDP-QA-03.

5.3.3.1 The expected results of QC control cultures after a minimum of 30 hours of incubation should conform to those shown below:

QC Culture	Expected Results
Uninoculated media	no growth, no blue color & no fluorescence
E. coli	growth, blue color & fluorescence
E. aerogenes	growth, no blue color to light blue color & no fluorescence
Produce control	growth, blue color & fluorescence

5.4 *E. coli* Test, LST- based MPN Method

5.4.1 Use aseptic technique throughout this SOP.

5.4.2 Resuspend the contents wash by shaking. Transfer 10mL of wash eluate into each of three test tubes containing 10mL DS LST.

5.4.3 Transfer 1mL of the wash into each of 3 tubes of 10mL SS LST.

5.4.4 Transfer 0.1mL of the wash into each of 3 tubes of 10 mL SS LST.

5.4.5 Refrigerate at least 15 mL of the wash to repeat test if necessary. Alternatively, the entire sample may be refrigerated.

5.4.6 Aseptically add two ColiComplete discs to each tube in 5.4.2 and one ColiComplete disc to each tube in 5.4.3 and 5.4.4 using sterile forceps.

5.4.7 Incubate tubes for 30-48 hours at 35 $\pm$ 2^{o}C.

Note: The tubes should be incubated for a minimum of 30 hours but can be incubated longer (overnight) and observed the following morning. Given the nature of produce, this practice will help low numbers of E. coli cells present in most samples to grow and give a definitive fluorescence indicating presence of E. coli.

5.4.8 After incubation visually examine the discs and the surrounding medium in the tubes for blue color under normal (visible range) light. The intensity of blue color may vary, but any blue reaction indicates a positive result for the presence of coliforms, including *E. coli.* Samples that fail to produce blue color on the discs should be recorded as negative.

5.4.9 For tubes that exhibited positive reaction for coliforms under visible light, examine the discs and the surrounding medium under long-wave (366 nm) ultraviolet (UV) light in the dark. A blue fluorescence indicates a positive reaction for the presence of *E. coli*. Record results.

5.4.10 Calculate the MPN of *E. coli* based on the number of LST tubes in which the discs and/or the surrounding media have fluoresced blue under the UV light indicating the presence of *E. coli*. See the attached table to determine the MPN. Results will be reported as MPN/mL of eluate with the understanding that all melons are processed using a volume of BPW + 0.1%Tween 80 equal to ¼ the weight of the individual melons. Normalization of MPN data for cantaloupe will be performed by MPO.

Table. MPN index and 95% confidence limits for various combinations of positive results using 3 tube dilution.. (Dilutions 10, 1.0, and 0.1 g)[a]

Combination of Positives	MPN index/mL	95% Confidence Limits		Combination of Positives	MPN index/mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.030	0.000	0.095	2-2-0	0.211	0.045	0.425
0-0-1	0.030	0.002	0.096	2-2-1	0.276	0.087	0.945
0-1-0	0.031	0.002	0.107	2-2-2	0.348	0.087	0.945
0-1-1	0.061	0.012	0.181	2-3-0	0.286	0.087	0.945
0-2-0	0.062	0.012	0.181	2-3-1	0.360	0.087	0.945
0-3-0	0.094	0.036	0.038	3-0-0	0.231	0.046	0.945
1-0-0	0.036	0.002	0.181	3-0-1	0.385	0.087	1.050
1-0-1	0.072	0.013	0.182	3-0-2	0.636	0.168	1.830
1-0-2	0.11	0.036	0.038	3-1-0	0.427	0.090	1.830
1-1-0	0.074	0.013	0.203	3-1-1	0.749	0.169	2.000
1-1-1	0.112	0.036	0.380	3-1-2	1.150	0.370	4.250
1-2-0	0.114	0.036	0.420	3-1-3	1.60	0.40	4.20
1-2-1	0.154	0.045	0.420	3-2-0	0.933	0.181	4.250
1-3-0	0.16	0.045	0.420	3-2-1	1.490	0.370	4.250
2-0-0	0.092	0.014	0.375	3-2-2	2.150	0.400	4.270
2-0-1	0.143	0.036	0.420	3-2-3	2.90	0.90	10.000
2-0-2	0.20	0.045	0.420	3-3-0	2.400	0.420	10.000
2-1-0	0.147	0.037	0.420	3-3-1	4.620	0.900	20.000
2-1-1	0.205	0.045	0.420	3-3-2	11.000	1.800	41.000
2-1-2	0.27	0.087	0.094	3-3-3	>11.000	4.250	--

[a]Modified from: Standard Methods for the Examination of Water and Wastewater, 14th ed., APHA AWWA-WPCF (1975), USDA/FSIS, Microbiology Laboratory Guidebook, 3rd ed., 1998, FDA/CFSAN -- BAM Appendix 2: Most Probable Number from Serial Dilutions, Bacteriological Analytical Manual. 1984, Most Probable Number Determination. 6th ed. BAM, Arlington, VA.

6. Preparation of Positive Cultures for Shipment

6.1 Storage of LST Broth in Glycerol

6.1.1 For each positive produce sample (based on fluorescence under UV light), label 2 sterile vials with sample number.

6.1.2 Aliquot 0.1mL sterile glycerol into each vial.

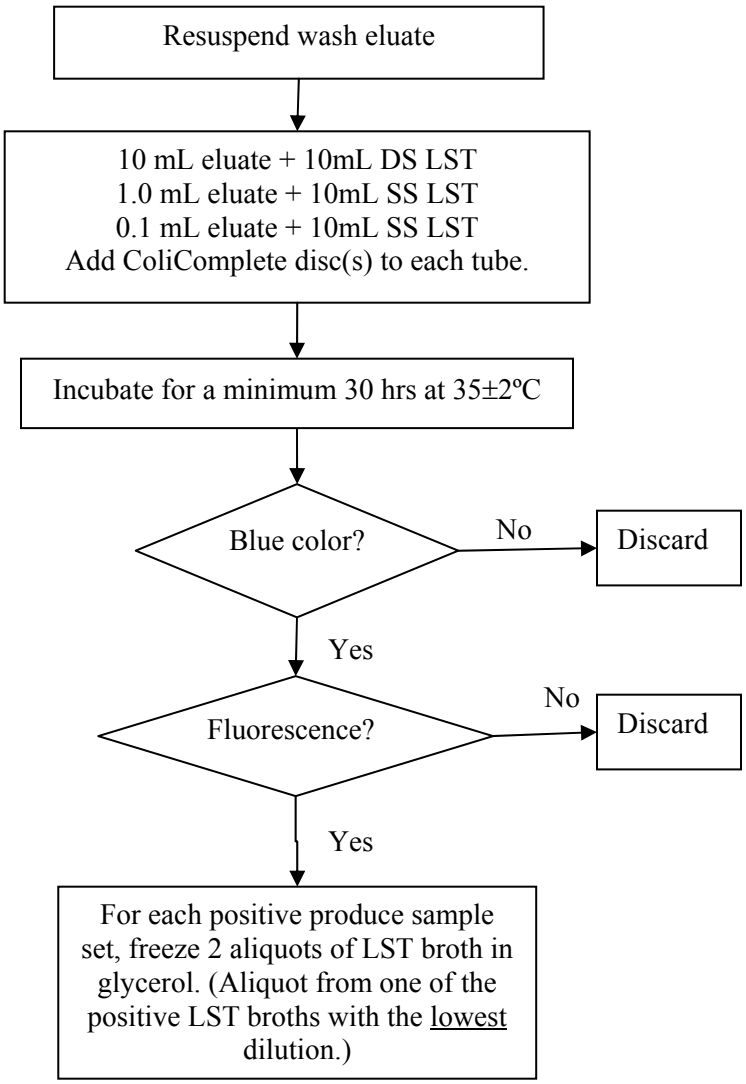
6.1.3 From the positive produce sample tube with the lowest dilution, add 0.9mL of LST culture broth to each of 2 vials.

6.1.4 Replace cap and mix by inverting a few times.

6.1.5 Store both vials at -70ºC or lower.

6.1.6 Record the date the vial was inoculated and frozen.

6.2 Refer to SOP MDP-SHIP-01 for shipment procedure and MDP-SHIP-02 for shipment locations and schedules.


Resuspend wash eluate
10 mL eluate + 10mL DS LST
1.0 mL eluate + 10mL SS LST
0.1 mL eluate + 10mL SS LST
Add ColiComplete disc(s) to each tube.
Incubate for a minimum 30 hrs at 35±2ºC
Blue color?
No
Discard
Yes
Fluorescence?
No
Discard
Yes
For each positive produce sample set, freeze 2 aliquots of LST broth in glycerol. (Aliquot from one of the positive LST broths with the lowest dilution.)

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Revision 03	Mar. 2004	MPO

- Changed from gas production and high temperature method to ColiComplete discs
- Changed procedure to ship LST broth in glycerol for additional testing instead of isolated colonies.
- New formatting applied